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Ultrastructural Studies and Transendothelial
Resistance of Phosgene-exposed
Endothelial Cells

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ABSTRACT

Sheep pulmonary artery endothelial cells (PAEC) were grown on Millicell CM™
inserts pre-treated with type IV collagen. Using these inserts we successfully
produced contiguous sheets of cells that mimic *in vivo* organization of endothelial
tissues. Ultrastructural details, obtained by transmission electron microscopy
showed that the plasmalemma of adjacent cells were partially joined by tight
junctions and overlapping end processes. They were also joined by compressed
basal lamina which lined the dorsoventral surfaces of apposing cells. In control
populations, the basal lamina were uniform in dimension (0.1 to 0.35 μm) along
their entire length and were filled with compressed extracellular matrix materials.
Transendothelial resistance of these contiguous endothelial populations was low and
averaged only 6.77 +/- 0.14 ohms per cm^2. Exposure to phosgene (137 ppm x 20
min) tore the basal lamina apart and produced large paracellular leakage paths.
Doses as high as 561 ppm x 20 min. had no obvious effects on other ultrastructural
details and did not decrease cell viability but did produce a 23% decrease in
transendothelial resistance. We conclude that phosgene's effects on the basal
lamina of PAEC may be linked by f-actin lesions to increased permeability and to
cytoskeletal-membrane interactions that control normal barrier function.

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INTRODUCTION

It has been known since the beginning of the century that the primary cause of death from phosgene inhalation is lung edema (Roos, 1914). The consequences of that fact were realized in the trenches of World War I where phosgene had devastating effects on military personnel and gave rise to extensive case studies on battlefield casualties and on the pathogenesis of phosgene toxicity. By 1978, phosgene was being commercially manufactured in million-ton volumes and concern about human exposure had been revitalized. However, despite occupational and military risks from exposure to phosgene, the mechanisms that disrupt barrier function and control permeability of pulmonary tissues remain poorly understood. Werrlein et al. (1991) have demonstrated that phosgene, in doses equivalent to 0.5, 1.0, 2.0 and 5.0 x LCt_{50} for sheep (3300 ppm · min), disrupts barrier function of sheep pulmonary artery endothelial cells (PAEC) grown in culture on cytodex-3 microcarrier beads. Dose-response effects from those studies indicated that the PAEC lesions produced by phosgene were peracellular. To confirm or refute that concept, we have investigated the effects of phosgene on transendothelial resistance and ultrastructure using the following *in vitro* model.

MATERIALS AND METHODS

Sheep Pulmonary Artery Endothelial Cells (PAEC) Cultures

PAEC from proliferating, low passage (4-7), stock cultures were seeded at 20,000 cells/cm^2 onto 12 mm Millic. I CM^{TM}, PTFE inserts (Millipore Products, Bedford, MA). Inserts (Fig. 1A) were pre-coated with type IV collagen (Sigma Chemical Co., St. Louis, MO). Attached, PAEC cultures were subject to medium renewal 3 times per week (M-W-F) and were maintained in a Hotpack incubator (Hotpack Corp., Phila., PA) at 35 °C in an atmosphere of 5% CO_2 and air.
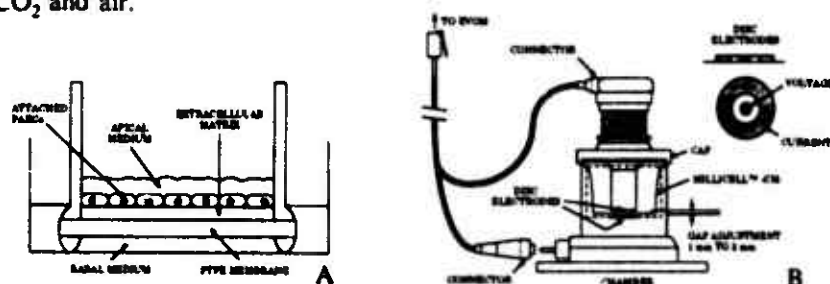

Fig. 1 Millicell CM^{TM} insert (A) showing PTFE Biopore^{TM} membrane, extracellular matrix of type IV collagen, layer of attached PAECs and surround of nutrient medium (apical and basal). Resistance measurement chamber (B) showing EVOM^{TM} connectors, position of the Millicell CM^{TM} insert relative to disc electrodes which face each other from opposite sides of the PAEC tissue sheet. Clearance between the top electrode and PTFE membrane was set at 1-2 mm.

Nutrient Medium

PAEC were grown in HAM's F-12 medium containing 15% fetal bovine serum, 5% Omni^{TM} serum (Advanced Biotechnologies Inc., Columbia, MD), L-glutamine (2mM), gentamicin sulfate (50μg/ml) and endothelial cell growth supplement (50 μg/ml). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO., unless otherwise stated.

Transendothelial Resistance Measurements

Transendothelial tissue resistance was measured with an EVOM^{TM} voltohm meter and an Endohm 12^{TM} resistance measurement chamber (Fig. 1B), World Precision Instruments, Inc., New Haven, CT. Current passed between apposing faces of disc electrodes, placed above and below the attached sheet of endothelial tissues, was used to determine resistance to the current flow. The background resistance of a blank (collagen coated insert without cells) was subtracted from culture readings and the difference used to determine resistance of the cell sheet. Normalized values were expressed as ohms $(\Omega) \cdot cm^2$.

Phosgene Exposure

To expose Millicell-CM^{TM} insert cultures, the overlaying, apical layer of medium was first reduced to a volume of 25 μl. That volume was sufficient to keep the PAEC wet and assure interaction with phosgene during the 20 min. dosing period. Immediately prior to exposure, replicate insert-cultures were placed in open, 60 mm Petri dishes containing 1.5 ml of freshly prepared medium. Replicates were then transferred to an environmentally controlled, 37 °C chamber and were exposed to a dose (137 ppm x 20 min) of phosgene in a humidified atmosphere of 5% CO_2 and air. The dose was verified by removing 10 μl gas samples from the exposure chamber at the beginning and end of each exposure and analyzing the sample by gas chromatography. Sham-treated control cultures were exposed to 5% CO_2 in air. At the end of an exposure period, replicate cultures were immediately prepared for transendothelial resistance and ultrastructural studies.

Ultrastructural Studies

Sham-treated controls and phosgene-exposed populations were rinsed with 0.1 M sodium cacodylate buffer then fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer. The cultures were washed again in 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide. After dehydration by passage through increasing alcohol gradients to propylene oxide, the cells were embedded in POLY/BED 812 (Polysciences Inc., Warrington, PA). Thin sections were cut on an LKB Ultramicrotome IV and stained with uranyl acetate and lead citrate.

RESULTS

Phosgene's Effects on Ultrastructure

Ultrastructural details show the major difference between sham-treated controls and phosgene-exposed populations was in tissue continuity. Untreated controls (Fig. 2A) had no obvious gaps between plasma membranes or apposing faces of adjacent cells. These populations were closely knit and cells that overlapped were held together by a thin scaffolding of compressed basal lamina. The basal lamina were uniform in dimension and defined an intercellular space of 0.1 to 0.35 μm along their entire length. That space was filled with a ground substance of extracellular

Fig. 2 Ultrastructure of control (A) and phosgene exposed (B) PAECs. The plasmalemma of control cells were held together by a thin scaffolding of compressed basal lamina (BL). BL were typically 0.1 to 0.35 μm wide along their entire length and were filled with a ground substance of extracellular matrix materials. When exposed to phosgene (137 ppm x 20 min), the basal lamina were rended apart and paracellular leakage paths (PLP) developed between the apposing faces of plasma membranes and cells that overlapped. Membranes joined by tight junctions (M) were not disrupted by phosgene.

matrix materials. In contrast, the basal lamina of sheep PAECs exposed to 2740 ppm min phosgene (Fig. 2B) were rended apart in a manner that separated the faces of apposed plasma membranes and produced large paracellular leakage paths. Cells from the exposed populations were slightly misshapen by the effects of phosgene but their organelles, e.g., nuclei, mitochondria, lysosomes and endoplasmic reticulum remained intact. Unions in

which plasma membranes were joined by tight junctions were resistant to separation by phosgene. Such unions frequently involved plasma membranes from adjacent cells that overlapped in thin folds at their dorsal and ventral edges.

Transendothelial Resistance

Populations of PAECs became confluent on Millicell CM^{TM} inserts within 1 week of seeding. However, we found these cells to be poorly suited for resistance studies. Even when confluent for 3-4 weeks, the average transendothelial resistance was only 6.77 $+/-$ 0.14 ohms $\cdot$ cm^2. All studies, therefore, required populations that were confluent for at least 4 weeks. High doses of phosgene did have a limited impact on transendothelial resistance but how these changes relate to barrier function is uncertain. Populations exposed to 11,220 ppm $\cdot$ min (561 ppm x 20 min) showed a progressive 10% to 23% decrease in transendothelial resistance relative to sham-treated controls (Fig. 3) during the ensuing 24 hour period. Results from smaller doses (2740 ppm $\cdot$ min) indicate that phosgene induced decreases in tissue resistance did persist for up to 72 hours (results not shown).
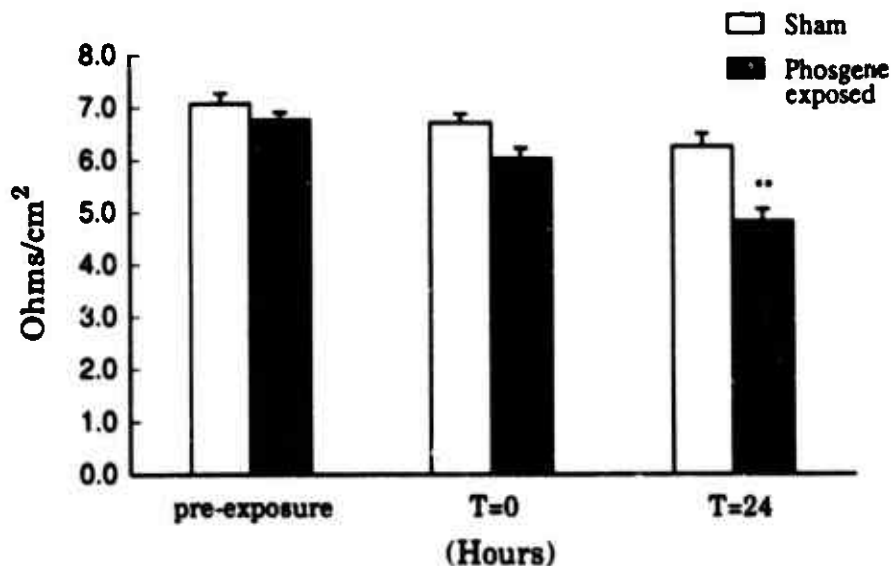

Fig. 3 Transendothelial resistance measurements of sheep PAECs in Millicell CM^{TM} insert cultures. Sham-treated control populations (white bars) were exposed for 20 min to 5% CO_2 and air. Populations exposed to phosgene (561 ppm x 20 min) are shown as black bars. Average readings (ohms/cm^2) and standard error bars compare replicate control (n = 9) and exposed (n = 17) populations at T = 0 hours (immediately after 20 min. exposure) and T = 24 hours post exposure. Results were subject to one way ANOVA ** = p < .01.

DISCUSSION

Our ultrastructural studies indicate that doses of 137 ppm x 20 min disrupted basal lamina and produced paracellular leakage paths in Millicell CM^{TM} cultures of sheep PAECs. These leakage paths are consistent with results from earlier studies (Werrlein et al., 1991) which showed that acute, 20 minute exposures to phosgene increased permeability of PAEC tissues immediately and in a dose-dependent fashion. Permeability was quantified in the 1991 study by transendothelial adsorption of erythrosin-B (a red viability stain) as it moved across the PAEC tissue barrier and adhered to the cell substrate, i.e., microcarrier beads. These direct and immediate effects of phosgene do not explain delayed onset of acute pulmonary edema, i.e., the "clinical latent phase." They may, however, help to explain why phosgene exposure in rats causes immediate increase in lung lavage fluid protein (Ghio et al., 1991). It is also interesting that F-actin depletion and paracellular leakage paths appear to develop in concert. That observation is in agreement with evidence which indicates that cellular interaction with basal lamina is F-actin dependent (Svorboda and Hay, 1987).

Average transendothelial resistance of sheep PAECs was very low (6.77 +/- 0.14 ohms · cm^2). Phosgene, in high doses (11,220 ppm min) did produce a progressive decrease in tissue resistance. However, to a lesser extent, so did manipulation of the sham-control populations. Significant decreases in resistance were observed in exposed populations at T = 24 hours. While our resistance values are in agreement with the literature where pulmonary transendothelial resistance ranges from 6.1 +/- 0.5 Ω· cm^2 to 15 Ω· cm^2 (Territo et al., 1984; Shasby and Shasby, 1986; Naveb et al., 1986), the low resistance of PAECs does not lend itself to sensitive and routine testing for toxicity or decreased barrier function. The model does, however, have considerable potential for *in vitro* study of lung cells and tissues, especially the effects of toxic gases on ultrastructure and barrier function.

CONCLUSIONS

Phosgene disrupted basal lamina of PAECs (in culture) and produced large paracellular leakage paths that indicate a mechanism for decreased barrier function.

Average transendothelial resistance of sheep PAECs is low (6.77 +/- 0.14 Ω· cm^2). High doses of phosgene (11,220 ppm· min) caused a small but progressive and significant decrease in tissue resistance during a 24 hour post exposure period.

Phosgene's effects on the basal lamina and F-actin cytoskeleton of PAECs may work in concert to increase permeability of the lung's endothelial tissues.

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